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Keys to solving health problems in small ruminants: anthelmintic resistance as a threat to sustainable nematode control.

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Highlights

- The focus of veterinary nematode control in intensively managed sheep flocks and goat herds must shift from attempts to eliminate parasite populations, towards the adoption of management and anthelmintic drug treatment strategies aimed at maintaining adequate standards of health in the face of a low level of challenge.
- The publication of a draft genome and transcriptome for the model nematode parasite, *Haemonchus contortus*, affords opportunities for post genomic research to identify sensitive molecular markers to evaluate resistance management strategies and potential candidates for novel control methods.
- The opportunity that now exists for collaboration between veterinary practitioners and research scientists to apply state of the art technologies for post genomic research to identify solutions to sustainable small ruminant production in field situations is unprecedented and exciting.

ABSTRACT

The epidemiology of nematode parasites has changed as they have adapted to climatic and management changes and as a consequence of the inappropriate use of anthelmintic drugs. This adaptability is conferred by large, polymorphic genomes and r-reproductive strategies. A significant net effect of these factors has been the emergence of anthelmintic resistance. Consequently, blueprint control programmes have failed and suboptimal sheep productivity due to nematode parasites has become commonplace. The focus of veterinary nematode control in intensively managed sheep flocks and goat herds must shift from attempts to eliminate parasite populations, towards the adoption of management and anthelmintic drug treatment strategies aimed at maintaining adequate standards of health in the face of a low level of challenge. Conventional parasitological methods are useful for the diagnosis of disease and for monitoring of nematode management over time, but they lack the sensitivity needed to mitigate effects of climate and management on population genetics of the parasites. The publication of a draft genome and transcriptome for the model nematode parasite, *Haemonchus contortus*, affords opportunities for post genomic research to identify sensitive molecular markers to evaluate resistance management strategies and potential candidates for novel control methods.

Keywords: Sheep Nematode Genomics Anthelmintic resistance Sustainability

1. Introduction: the importance of small ruminant production

Small ruminant farming has an important role to play in meeting the challenge of sustainable global food security. It is necessary to consider small ruminant production as part of a global food producing industry, and to acknowledge that efficient production from each sector of this industry is essential to meet the needs of the worlds growing human population that currently exceeds 7 billion. When addressing production efficiency, resources must be used in a manner that minimises environmental footprints, meets high standards of animal welfare and is socially acceptable. It is therefore necessary to understand and address: effects of population growth itself; demands of urbanisation and affluence; consequences of climate change; and hitherto irresponsible use of drugs and chemicals. These considerations are equally pertinent to the control of helminth parasites, which include many of the primary animal health constraints to small ruminant health and production. For example: population growth itself increases the need to manage disease to achieve more efficient food production from reducing resources; affluence associated with urbanisation increases demands for luxury food products such as sheep and goat meat, while reducing the land area for production; climate change has a profound effect on pathogens with free living stages in the environment; while the hitherto irresponsible use of medicines has resulted in the emergence of antimicrobial and anthelmintic drug resistance.

Goats are important because they naturally feed by browsing, therefore utilising poor quality nutrients to produce food, but are also suited to being kept under intensive or semi-intensive grazing or conserved forage management systems. Sheep are important due to their ability to convert short pastures efficiently into meat. Different breeds and types of small ruminants have been adapted and selected to suit specific climates and environmental resources and their potential as a means of alleviating poverty in diverse, often seasonally resource-poor, environments is well recognised (Pollott and Wilson, 2009). However, this potential is seldom realised, due to failure to translate research and development of sustainable husbandry and health management, in particular helminth parasitism, into efficient utilisation of natural resources.

2. Nematode (roundworm) parasites as the foremost constraint to economic small ruminant production

Nematode parasites are amongst the most important production-limiting diseases of ruminant livestock worldwide. *Teladorsagia circumcincta*, *H. contortus*, *Trichostrongylus vitrinus/colubriformis* and *Nematodirus battus* are of particular relevance. These parasites cause a range of diseases in their hosts, from diarrhoea to anaemia, and cause significant economic losses to farmers and their keepers in terms of reduced production and treatment costs, as well as being a major welfare issue for the infected animals. They also reduce production efficiency, thereby potentially raising food prices and damaging the environment. Gastrointestinal trichostrongyle nematodes have direct life cycles, involving non-parasitic environmental egg, first, second and third stage larvae, and parasitic third and fourth stage larvae, and dioecious, sexually reproducing adults. The biology of these parasites is, therefore, influenced by both environmental and host factors, which must be accounted for when planning for their control.

The aim of all approaches to nematode control is to limit the host challenge to a level which does not compromise performance or welfare, while at the same time enabling the development of protective immunity. Sustainable nematode control in individual flocks or herds must, therefore, be underpinned by knowledge of the farming system and of the relationship between pasture contamination, the availability of infective larvae and the build up of infection in animals. For example, a health plan for nematode control in lambs which will be grazed on a particular field during the summer must include consideration of:

consequences of prior autumn and winter grazing management and climate on the size of the overwintered infective larval population; potential influences of anthelmintic drug treatments of periparturient ewes, ewe nutrition and the lambing percentage on ewe faecal egg output onto that field; and effects of anthelmintic drug treatments, nutrition and grazing management on autoinfection of the field by the lambs themselves. In the face of the economic impact of nematode parasites and the complexity of the principles underpinning their control, most farmers and ruminant livestock keepers, worldwide, rely upon the use of anthelmintic drugs in an unsustainable attempt to eliminate the parasites.

3. Sustainable roundworm control

Nematode control is compromised by the parasites' adaption to climatic and management changes and hitherto irresponsible use of anthelmintic drugs. Nematode parasites have large genomes, with large numbers of genes and extraordinarily high levels of polymorphism and r-reproductive strategies giving rise to high biotic potential. The latest assembly of the *H. contortus* genome is about 320 mb, with about 22,000 protein coding genes (Laing et al., 2013), while each female can shed more than 4,000 eggs per day (Sargison, 2009). These parasites will inevitably evolve in response to both favourable and hostile conditions afforded by effects of climatic or management changes on free-living stages and exposure of parasitic stages to anthelmintic drugs, respectively.

Suboptimal sheep productivity due to nematode parasites has become commonplace in small ruminant flocks and herds during recent years, despite the adoption of previously highly successful control programmes involving the use of anthelmintic drugs. Clinical investigation of these problems and parasitological monitoring show that nematode parasite epidemiology now differs from the conventional perspective in various aspects, giving rise to unexpected scenarios such as spring teladorsagiosis in young lambs caused by high levels of overwintered infective larvae on pasture (Sargison et al., 2002); autumn nematodiosis due to the prolonged survival of third stage larvae in particular environmental niches (Sargison et al., 2012); and haemonchosis associated with development of free living stages of the parasite during opportunities presented by warm spring or autumn weather (Sargison et al., 2007; Falzon et al., 2013). These problems have arisen because the epidemiology of the parasites has changed due to a combination of interacting factors. These include: new strategies in farm and grazing management caused by the changing economics of sheep production; host immune mechanisms in response to infective larval challenge; parasite evolution; and micro- and macro-climatic variation (Kenyon et al. 2009a; Van Dijk et al. 2010). Consequently the nature and timing of prescriptive nematode evasive management or anthelmintic treatments may be inappropriate. A significant net effect of these factors has been the inevitable emergence of anthelmintic resistance. Nematode control is therefore unsustainable and the immediate challenge facing small ruminant farmers and keepers is to ensure that current measures enable economically viable sheep production for long enough to allow for the development of new strategies before the existing methods eventually fail altogether.

While fully sustainable nematode control is not possible, acceptable compromises based upon an improved understanding of the parasites' biology and detailed relevant knowledge of individual farming systems are achievable. Advances are needed in these areas to validate the interpretation of the relationships between pasture contamination, the availability of infective larvae on pasture and the accumulation of infection in sheep. Planned investigation and monitoring of animal performance is important, in order to identify and understand those conditions, management practices, aspects of parasite biology and parasite population genetics that have been adopted or have arisen to enable sustainable productivity, for example, in the face of anthelmintic resistance. Thus, iterative small ruminant flock and herd health planning is an essential first step towards sustainable nematode control.

4. Anthelmintic resistance management

Following reports of *T. circumcincta* resistance to benzimidazole, imidazothiazole and macrocyclic lactone anthelmintic groups (Sargison et al., 2001; Sargison et al., 2010), the potential threat to future health and profitability of European small ruminant production is recognised, and the need for management combining effective nematode control with minimal further selection for resistance is acknowledged. Globally, anthelmintic resistance has now been reported in most small ruminant parasitic nematode species, including *N. battus* (Morrison et al., 2014) and to most drugs, including monepantel (Van den Brom et al., 2015). Advice is currently given based on the premise that alleles conferring anthelmintic resistance are already present in most sheep flocks. This can be summarised as: i) ensuring that the nematode parasites are exposed to an effective anthelmintic drug concentration; ii) considering the timing and frequency of anthelmintic drug treatments so that only a small proportion of the population is exposed to the anthelmintic; iii) treating introduced animals with effective anthelmintic drugs; and iv) monitoring for anthelmintic resistance. This advice is based entirely upon theoretical principles, hence its validity is unknown. For example: the extent to which underdosing selects for anthelmintic resistance in field situations, and whether or not the practice selects for the same mechanisms of resistance as pressures relating to the timing and frequency of drug treatments is unknown; there is a paucity of empirical knowledge pertaining to different management systems and concerning the influences of timing and frequency of treatments and proportions of animals treated on the efficacy of strategies aimed at only exposing a small proportion of the parasite population to the anthelmintic drug; and the extent to which treatment of introduced animals with effective anthelmintic drugs influences the balance of flow between genes conferring anthelmintic resistance and susceptibility is unknown. Thus, the most important recommendations are arguably those involved with monitoring of parasitism and anthelmintic resistance. Nevertheless, in the absence of contradictory research findings concerning the population genetics of resistant nematodes, the recommendations are pragmatic, and considerable effort has been placed on their dissemination to small ruminant farmers and keepers. Unfortunately, uptake of some of these recommendations has been poor, in part due to the complexity and superficial impracticality of what have been perceived as being mixed messages concerning the timing and frequency of anthelmintic drug treatments.

Experience of the emergence of triclabendazole resistance in *Fasciola hepatica* in the UK and Republic of Ireland, where subacute fasciolosis is an important cause of production loss that can only be effectively treated using triclabendazole, has heightened farmer awareness of the principles of evasive management strategies, or strategic use of drugs with the aim of reducing the challenge to the host rather than eliminating the parasite (Sargison and Scott, 2011). The focus of veterinary nematode parasite control in all intensively managed sheep flocks, not only in those where the high prevalence of anthelmintic resistance is known, must now move away from attempts to eliminate parasite populations, towards the adoption of management and strategic application of anthelmintic drug treatments. These are aimed at maintaining adequate health standards in the face of a low level of challenge; for example, involving evasive grazing management and drug treatments targeted towards individual animals (Greer et al., 2009; Kenyon et al., 2009b; Busin et al., 2014) while leaving others untreated as a source of *refugia* (Van Wyk, 2001). In summary, the theoretical principle underpinning this concept is that once the numbers of parasite stages in a refuge from drug exposure (*in refugia*, either in the environment, or as hypobiotic stages within their host) is reduced to a low level, then the progeny of parasites surviving treatment of their hosts contribute to a significant proportion of the subsequent total parasite population. Thus, if the parasites survive treatment due to their being genetically drug resistant, the frequency of resistant nematodes in the total population increases, followed by an increase in the size of the parasite population as subsequent anthelmintic treatments are ineffective, and eventually leading to disease outbreaks that cannot be controlled using anthelmintic drugs.

5. Conventional parasitological tools for the diagnosis and monitoring of anthelmintic resistance

Our understanding of the biology and epidemiology of nematode parasites is underpinned by the use of conventional parasitological tools. Faecal egg counts (FECs) provide valid information about the presence of patent nematode infections, but the value of information concerning numbers of eggs per gram (epg) of faeces is limited by the subjectivity of their interpretation. FECs are a ratio of numbers of eggs to weight of the faeces, hence their interpretation depends upon knowledge of the relative faecal dry matter content, feed intake and the manner in which the animals were fed at the time of sampling. In turn, the faecal dry matter may be influenced by host responses to nematode parasitism (Colditz, 2008), altering the epg ratio, but with no overall effect on the total number of eggs shed. It is also necessary to take into consideration the variation of egg production in relation to the numbers and pathogenicity of adult female nematodes of different species and temporal host regulatory influences on egg production of female nematodes (Stear et al., 1995). The eggs of *Teladorsagia*, *Haemonchus*, *Trichostrongylus* and *Cooperia* are not easily distinguishable without resorting to morphometric analysis, fluorescent agglutinin staining, molecular methods (Gasser, 2001) or morphological identification of third stage larvae (L₃) recovered from coprocultures (Crilly and Sargison, 2015). The main applications of FECs and conventional speciation methods are the diagnosis of nematode parasitism during the investigation of disease, and monitoring of nematode management over time (Sargison, 2013). Within these contexts, FECs must be interpreted in conjunction with knowledge of farm management, parasite control practices, judgment of the parasites that are likely to be present, and knowledge of climate and local geography.

While FECs have been pivotal to our understanding of changes in nematode parasite epidemiology, the immediate challenge is to prevent production loss in the face of inevitable parasite evolution. Parasites will adapt to effects of changing biotopes on their free-living stages and adverse conditions, such as anthelmintic drug exposure, for their parasitic stages. Faecal egg count reduction tests, when combined with morphological identification of third stage larvae recovered from pre- and post-treatment coprocultures provide a useful indication of the presence of anthelmintic resistance (Coles et al., 2006), but are inaccurate in determining the true population prevalence of resistance conferring alleles. Bioassays such as the egg hatch, larval development and larval feeding inhibition tests also provide an indication of the presence of resistance, but only allow for estimation of the prevalence of resistant nematodes in monospecific populations. Conventional parasitological tools are therefore inadequate as a basis for early detection and analysis of the extent of the problem that is needed in order to understand effects of climate and management on the origins and spread of resistance in different nematode species (Kaplan and Vidyashankar, 2012). There is therefore a need to integrate genomic research into clinical veterinary medicine and conventional parasitology to identify the molecular basis and population genetics of changing parasite epidemiology in order to inform effective management solutions (Kotze et al., 2014).

6. Genomic markers to validate practices aimed at slowing the emergence of anthelmintic resistance

Standard approaches to identify markers for the molecular basis of anthelmintic resistance involve the investigation of associations between the resistance phenotype and polymorphisms in candidate genes, selected on the basis of knowledge of the drug's mode of action. Once mutations have been identified within candidate genes, it is necessary to investigate genetic and biochemical differences between susceptible and resistant parasites, in order to obtain circumstantial evidence for the pharmacological relevance of altered proteins in conferring resistance. Studies are then required to compare levels of their transcription between resistant and susceptible nematodes. Functional studies of the effect of the mutation

on drug binding can then be used to prove a causal relationship between a mutation in a candidate gene and the resistance phenotype (Gilleard and Beech, 2007).

Candidate gene approaches depend upon prior assumptions about resistance mechanisms, which are poorly defined for the macrocyclic lactone class, and will fail if mechanisms of resistance are independent of mechanisms of drug action and consequently not anticipated based on knowledge of the molecule. The standard approach to demonstrating an association between mutations in candidate genes and phenotypic expression of resistance is to compare parasite populations that differ in their response to treatment. However, the extreme genetic variation that exists in *H. contortus* populations (Redman and others, 2008) means that genetic differences exist between the two isolates, regardless of any association with resistance. In this case, the final genetic analysis may reveal differences between the initial subpopulations that are related to the genetic diversity of the parent population, but not to the selected resistance mechanism. This problem might be surmounted by deriving susceptible and resistant lines from a single isolate by experimental selection for anthelmintic resistance (Coles and others, 2005) to produce significant phenotypic differences between treated and untreated groups. Since these lines share an initial genetic pool, then differences between selected resistant and parent susceptible lines might be attributed to anthelmintic resistance. However, a potential disadvantage of deriving susceptible and resistant lines from a single isolate by experimental selection for anthelmintic resistance, is that the approaches may select for characteristics of general fitness, rather than solely for anthelmintic resistance (Gilleard and Beech, 2007). Furthermore, experimental selection for anthelmintic resistance by *in vitro* pressurisation, or *in vivo* by underdosing may not involve the same mechanisms as field selection.

The candidate gene approach has been successful in showing polymorphisms in isotype-1 β -tubulin gene conferring benzimidazole resistance in various nematode species (Kwa et al., 1994), but has yet to unequivocally identify molecular loci responsible for resistance against other anthelmintic drug classes. Nevertheless, isotype-1 β -tubulin gene markers have proved to be useful in the study of the origins and spread of benzimidazole resistance in *H. contortus* in small ruminants, suggesting multiple independent origins of the F200Y (TAC) mutation and low genetic differentiation between populations, consistent with high gene flow among sites (Chaudhry et al., 2015). Pragmatic theoretical advice concerning slowing the emergence of anthelmintic resistance is based on the premise that resistance is conferred by a single genetic mutation, without reversion to susceptibility. While benzimidazole and possibly levamisole (Martin et al., 2012) resistance may be encoded by single nucleotide polymorphisms (SNPs) in a single gene, it is unlikely that this relatively straightforward situation will pertain in the case of resistance to macrocyclic lactone and possibly other drug groups. Failure to demonstrate associations between the macrocyclic lactone resistance phenotype and polymorphisms in candidate genes coding for potential mechanisms of drug action, such as ligand gated chloride channels (Dent et al., 2000) and P-glycoproteins involved in trans membrane drug efflux pumps (Blackhall et al., 1998), indicate the need for a neutral genetic approach to identify anthelmintic resistance markers.

One such approach that is not influenced by prior assumptions concerning resistance mechanisms has been to conduct genetic backcrosses between populations of field-selected susceptible and resistant *H. contortus*. These have resulted in the introgression of genomic regions linked to resistance alleles or genes in the resistant parent populations into a known susceptible isolate (Redman et al., 2012). Confirmation of the success of these backcross experiments using a panel of parasitological and genomic methods, demonstrates the feasibility of forward genetics to introgress genes between different strains of a parasitic nematode. The resulting fourth backcross populations are a valuable resource for comparative genomic studies to identify polymorphisms in neutral markers between the original susceptible population and the backcross populations carrying introgressed resistance alleles, in the iterative search for linkage to anthelmintic resistance markers. However, the

analysis of genetic crosses to identify markers for anthelmintic resistance requires a high quality genome and transcriptome, from which a large panel of mapped neutral loci can be derived.

The anthelmintic susceptible MHco3 (ISE) isolate of *H. contortus* was chosen as an appropriate model parasitic nematode for genome assembly and genetic approaches to identify loci conferring anthelmintic resistance because of the global economic importance of the parasite species and relative ease of generating experimental infections. However, preliminary attempts to assemble a genome from 20 fold coverage of overlapping contigs and scaffolds of genomic sequence were hindered by the high level of genetic polymorphism within the isolate. This problem was addressed by inbreeding the MHco3 (ISE) isolate of *H. contortus* using a single parent genetic crossing method, enabling assembly of a draft high-quality genome and transcriptome (Laing et al., 2013). The *H. contortus* genome will enable genome wide SNP analysis of differences between the backcross populations and their resistant and susceptible parents to show multiple genomic regions likely to be linked to the genes conferring macrocyclic lactone resistance.

The availability of a high quality *H. contortus* genome assembly affords exciting opportunities to investigate patterns of selection across the entire genome (Gilleard, 2013). Population genomics approaches can now be employed to identify genes determining complex phenotypes of relevance to nematode survival. This will enable the identification of molecular markers of anthelmintic resistance and candidates for novel nematode control methods. The next steps are: i) to undertake genome improvement and the provision of genome-wide population genetic markers through the creation of a genetic linkage map; ii) genetic analysis of the backcrosses; iii) further analysis of loci under selection and genes of interest in a *Caenorhabditis elegans* transgenic system; iv) association studies between levels of resistance and genotype; v) population genomics to identify drug resistance mutations in field isolates; vi) development of models of migration and gene flow within the parasite population; vii) modelling of resistance in farms with different anthelmintic usage patterns; viii) inform sustainable parasite control strategies on the farm; ix) a whole genome approach to vaccine candidate selection; and x) application of methods to other important nematode parasites of small ruminants or cattle in temperate climates, in particular *T. circumcincta* or *Ostertagia ostertagi*, respectively (BUG consortium, 2015).

7. Concluding remarks

The rate of improvement of genetic sequencing platforms has increased almost exponentially over recent years, while the cost of long-read and even whole genome sequencing has been reduced accordingly. The opportunity that now exists for collaboration between veterinary practitioners and research scientists to apply state of the art technologies for post genomic research to identify solutions to sustainable small ruminant production in field situations is unprecedented and exciting. The development of novel genetic crossing methods ought to allow for rapid advance in the understanding of anthelmintic resistance and effective implementation of strategies aimed slowing its emergence and spread.

Conflict of interest

The author reports no conflicts of interest regarding the information provided in this manuscript.

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